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North American Truffles in the Tuberaceae: Molecular and Morphological Perspectives

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Abstract: The truffle genus *Tuber* (*Ascomycota*, *Pezizales*, *Tuberaceae*) produces underground mushrooms widely sought as edible fungi. *Tuber* species are distributed throughout Northern hemisphere forests and form obligate ectomycorrhizal symbiosis with trees within the Pinaceae, Fagaceae, Betulaceae, and Juglandaceae. Of the approximately 100 species of *Tuber* worldwide, half are suspected to be endemic to North America. In this study we use multiple genetic loci to assess patterns of phylogenetic diversity within *Tuber* in order to infer species boundaries and to define morphological and phylogeographic species groupings. Seven major clades were resolved (*Aestivum*, *Melanosporum*, *Rufum*, *Canaliculatum*, *Gibbosum*, *Puberulum*, and *Maculatum*). Two morphologically distinctive species *T. asa* & *T. excavatum* were unresolved. The three most specious clades of *Tuber* (*Rufum*, *Puberulum*, *Maculatum*) are distributed across Europe, Asia, and North America and are comprised mainly of non-commercial species.

Key words: Taxonomy; *Tuber*; Phylogenetics
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Introduction

The ‘true truffles’ are Ascomycete fungi belonging to the genus *Tuber* and are distributed throughout the northern hemisphere. There are estimated to be around 100 species of *Tuber* worldwide, half of which appear to be endemic to North America. *Tuber*, belongs within the Tuberaceae, a family that includes northern hermisphere genera (*Tuber*, *Choiromyces*) and southern hemisphere genera (*Reddellomyces*, *Dinglyea*, *Labyrinthomyces*) (O'Donnell *et al.*, 1997).

Tuber species form ectomycorrhizal (EM) symbiosis with many important timber and nut tree species, including those belonging to the Pinaceae, Fagaceae, Salicaceae, Betulaceae, and Juglandaceae. These hypogeous fungi have coevolved with mammals, which are known to be important agents in the dispersal of truffle spores. Additionally, many European and a few North American *Tuber* species are prized edibles and have the potential to be lucrative agricultural crops.

The first valid description of the genus *Tuber* was by F. H. Wiggers (1780), according to the current International Code of Botanical Nomenclature (Greuter, 1999). Wiggers recorded a single species from north Germany, named it *T. gulosorum* and described it as (translated from Latin): “a subglobose fungus filled with tasty flesh.” While this description is of little taxonomic value, it meets the requirements of the Code (Trappe, 2001). The first useable descriptions of species of *Tuber* were by Vittadini (1831), whose collections representing most of his species are preserved in good condition at the University of Turin herbarium in Italy.

The taxonomic literature on North American *Tuber* spp. began with H.W. Harkness (1899) (Fig.1) and was expanded by Helen Gilkey (1916, 1939, 1947, 1954). Other useful contributions include those of Butters (1903), Trappe and Guzmán (1971), Trappe (1975, 1979), Uecker and Burdsall (1977), Cázares *et al.* (1992), Trappe *et al.* (1996), Colgan and Trappe (1997), Trappe and Castellano (2000), Trappe and Cázares (2000, 2006) and Frank *et al.* (2006). Fortunately, unlike many European species, all described North American species are represented by type collections. Currently, are recognized (Table 2).

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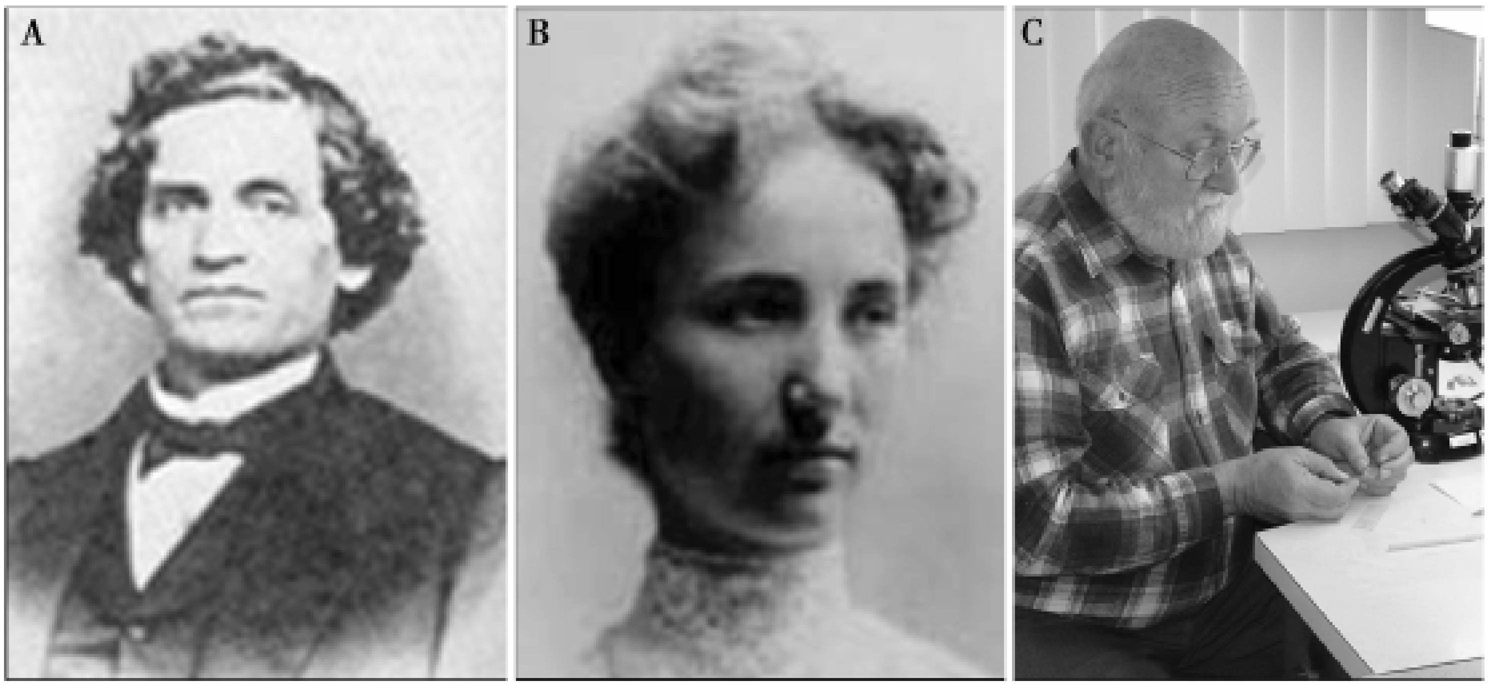

Fig . 1 Pioneers of North America Truffle Taxonomy

A . Taxonomy of North American truffles began with Dr . Harvey Harkness (1821 - 1901) . B . Dr . Helen Gilkey (1886 - 1972) described and illustrated many new species of North American truffles . C . Dr . Jim Trappe (1933-present) has described and revised truffle taxonomy and systematics throughout his career

At present, about 250 names have been applied to species and varieties in the genus *Tuber* . Many species have been named more than once by different authors . By eliminating probable synonyms, we estimate for Europe about 33 of species of *Tuber* are recognized (Table 1) (Berkeley and Broome, 1846; Ceruti *et al.*, 2003; Montecchi and Sarasini, 2000; Paolocci *et al.*, 2004) and 35 species of *Tuber* in North America . For Asia at least 11 species of *Tuber* have been validly described (Table 3), and an unknown and likely large number are still undescribed (Cooke and Massee, 1892; Wang *et al.*, 1998; Wang and He, 2002; He *et al.*, 2004, Zhang *et al.*, 2005; Hu and Wang, 2005; Chen *et al.*, 2005; Chen and Liu, 2007; Jeandroz *et al.*, 2008) . No endemic *Tuber* species are known from the southern hemisphere . Species of *Tuber* described from Australasia and South America have in all cases been associated with trees introduced from the northern hemisphere and probably came as hitchhikers on roots of imported seedlings (Trappe and Cázares, 2000) .

The main goal of this research is to define morphological and phylogeographic groupings within the Tuberaceae, particularly within species complexes and widely distributed taxa (e.g . *T. rufum* complex, *T. maculatum* complex) . In addition, using the resulting phylogenetic framework, we aim to place sequences of

Table 1 *Tuber* species described from Europe Species with commercial interest are labeled with an asterix (*)

Tuber species in Europe
* *T. aestivum* (= *T. uncinatum*) Vittadini
T. asa Tulasne & Tulasne
T. belionae Quelet
* *T. borchii* Vittadini
* *T. brumale* Vittadini
T. dryophilum Tulasne & Tulasne
T. excavatum Vittadini
T. ferrugineum Vittadini
T. foetidum Vittadini
T. fulgens Quelet
T. lutescens Lazaro & Ibiza
* *T. macrosporum* Vittadini
T. maculatum Vittadini
* *T. magnatum* Pico
T. malenconi Donadini, Riousset, Tiousset & Chevalier
* *T. melanosporum* Vittadini
T. mesentericum Vittadini
T. microsporum Vittadini
T. michailowskoanum Bucholtz
T. murinum Hesse
T. multimaculatum Parlade, Trappe & Alvarez
T. nitidum Vittadini
T. oligospermum (Tulasne & Tulasne) Trappe
T. pallidum Lazaro & Ibiza
T. panniferum Tulasne & Tulasne
T. puberulum Berkeley & Broome
T. queietianum Ferry de la Bellone
T. rapoeodorum Tulasne & Tulasne
T. regianum Montecchi & Lazzair
T. rufum Pico
T. scruposum Hesse
T. sinuosum Lazaro & Ibiza
T. verii Pacioni & Lalli

Tuber from ectomycorrhizal root tips and unidentified fruitbodies into this phylogenetic framework.

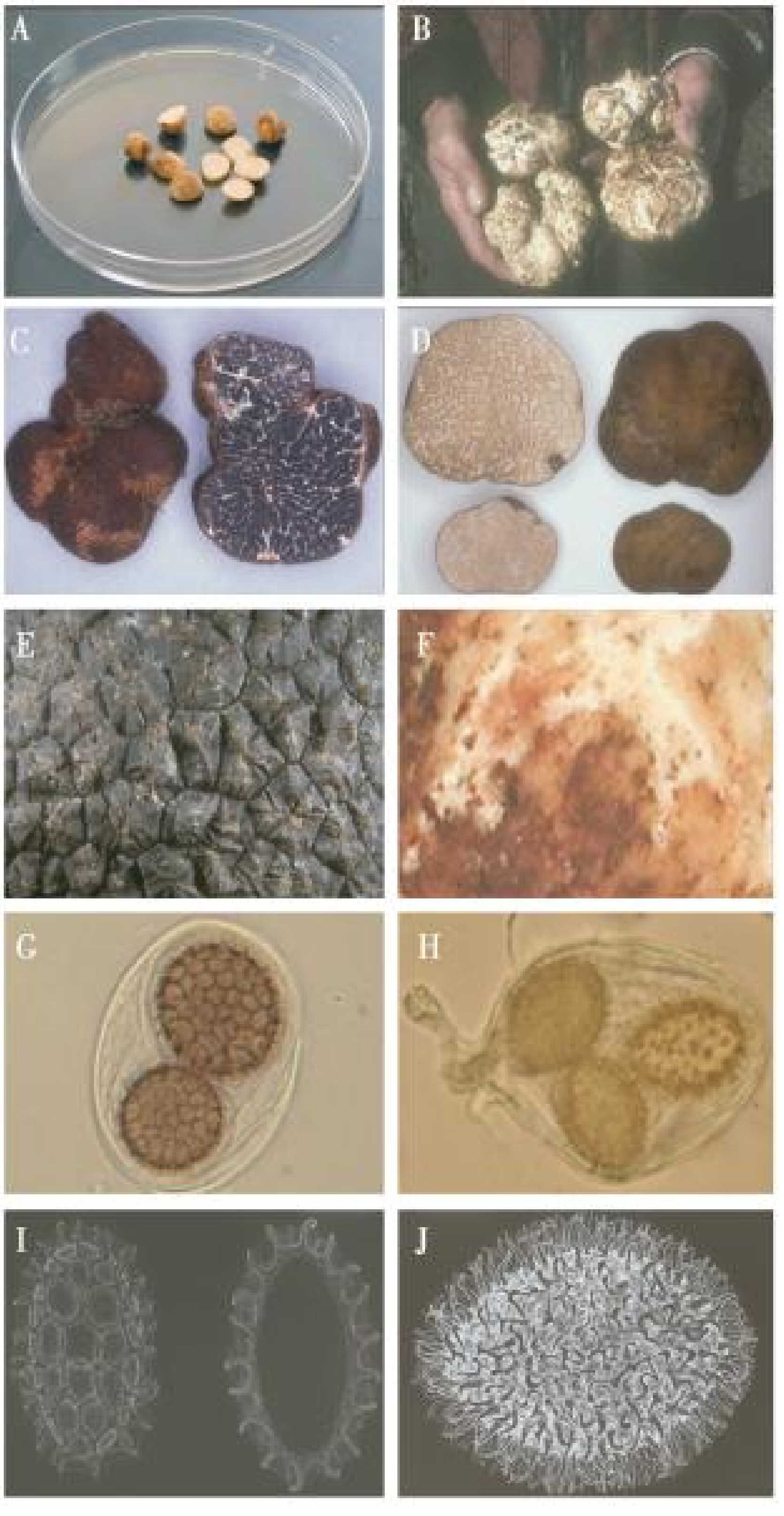

Fig. 2 Morphological Variation of *Tuber*

Truffles vary in size along a continuum from very small to very large, as exemplified by a collection of and unidentified Tuber (A) and *T. gibbosum*, respectivly (B). Tuber species are characterized by a pattern of sterile and fertile veins filling the gleba, which darken as the fertile tissue matures and vary in color by species as shown with *T. canaliculatum* (C) and *T. lyonii* (D). Other gross morphological features help to distinguish *Tuber* species, such as the texture of the peridium. The peridium may bear large warts as in *T. aestivum* (E), or it could be rough, scaley, pubescent, or glabrous as in *T. oregonense*. nom. prov. (F). Microscopic characters of the asci and spores are necessary for distinguishing species of *Tuber*. Asci may be globose or sub-globose as shown for unidentified *Tuber* (G) or more flask-shaped and bearing a stem as in *T. lyonii* (H). Finally, spores may be alveolate-reticulate or spiny, illustrated by *T. maculatum* (I) and *T. melanosporum* (J), respectively

Table 2 *Tuber* species described from North America Species with commercial interest are labeled with an asterix (*)

Tuber species in North America
T. anniae Colgan & Trappe
T. argenteum Gilkey
T. besseyi Gilkey
T. bellisporum Bonito & Trappe nom. prov.
T. californicum Harkness
* *T. canaliculatum* Gilkey
T. candidum Harkness
T. castellanoi Bonito & Trappe nom. prov.
T. citrinum Harkness
T. clarei Gilkey
T. gardneri Gilkey
* *T. gibbosum* = *T. giganteum* Gilkey
T. guzmanii Cazarez & Trappe
T. harknessii Gilkey
T. irradians Gilkey
T. levissimum Gilkey
T. linsdalei Gilkey
T. longisporum Gilkey
T. luomai Trappe nom. prov.
* *T. lyonii* Butters
T. monticola Harkness
T. murinum Hesse
* *T. oregonense* Trappe & Bonito nom. prov.
T. pacificum Trappe & Castellano
T. phleboderma (Gilkey) Trappe comb. nov.
T. quercicola Frank, Southworth, & Trappe
T. rapaeodorum Tulasne & Tulasne
T. regimontanum Guevara, Bonito & Rodríguez
T. separans Gilkey
T. shearii Harkness
T. sphaerosporum Gilkey
T. spinoreticulatum Uecker et Burdsall
* *T. texense* Heimsch
T. unicolor Gilkey
T. whetstonensis Frank, Southworth, & Trappe

Table 3 *Tuber* species described from Asia Species with commercial interest are labeled with an asterix (*)

Tuber species in Asia
T. formosanum Hu
T. furfuraceum Hu & Wang
T. huidongense Wang & He
* *T. indicum* Cooke & Massee
T. latisporum Chen & Liu
T. liatongense Liu
T. liui Xu
T. pseudoexcavatum Wang, Moreno, Riousset, Manjón & Riousset
T. taiyunense Wang
T. umbilicatum Chen & Liu
T. zhongdianense Wang

Methods

Morphological: *Tuber* spp. form stereothecia with a distinct, simple to layered peridium enclosing a gleba of fertile tissue marbled with sterile, hypha-stuffed

veins that tend to open through the peridium. The asci are randomly embedded in the fertile tissue and within a specimen may contain from one to 4 (-6) spores. The following character states have proven useful in differentiating species, in general order of importance. *Spores* spiny, spiny with a reticulum of low lines, or reticulate with tall reticular walls; ellipsoid to subglobose or globose; spores in 1- or 2-spored asci up to 45, 55, 65 or 75 μm long. *Asci* astipitate, with a short stipe, or a long stipe with a forked base; thin-walled or with walls up to 1, 2 or 4 μm thick. Ascomatal surface smooth, pubescent with outgrowing hyphae, pubescent with tapered cystidia, scabrous, verrucose with rounded warts, verrucose with angular warts, white, gray, yellow, olive, brown, reddish brown, dark brown or black. *Peridium* a single, undifferentiated layer or with two or more layers differentiated by size, shape, wall thickness, or pigmentation of the cells; greatly inflated cells absent or present in one or more layers. *Glebal* cells similar to those of inner peridium or differentiated by cell diameter or presence of inflated cells. Species can usually be differentiated by the large number of combinations of these various characters, but complexes of closely related species can be difficult to separate without supporting molecular data (Mello *et al.*, 2000; Halász *et al.*, 2005; Frank *et al.*, 2006).

Molecular: DNA was extracted with 24∶1 chloroform: isoamyl alcohol and precipitated in isopropanol. Both the internal transcribed spacer region (ITS1, 5.8S, and ITS2) and three divergent domains (D1, D2, D3) of the ribosomal large subunit (LSU) locus were amplified using the universal fungal primer set ITS5-LR5 (Bertini *et al.*, 1999; Vilgalys and Hester, 1990). Other loci from these extractions were PCR amplified, including the ribosomal small subunit (SSU), elongation factor 1 alpha (EF1α), the second subunit of RNA polymerase (RPB2). PCR conditions and the handling of PCR products were as described in Healy *et al.* (2009). Sanger sequencing was performed on an ABI3700 (Applied Biosystems, Foster City, CA) using Big Dye chemistry version 3.1 (Applied Biosystems, Foster City, CA) in both directions. DNA sequences were assembled and manually edited using Sequencher 4.0 (Gene Codes, Ann Arbor, MI) and queried against the NCBI public database GenBank (http: blast.ncbi.nlm.nih.gov Blast.cgi) with the BLASTN algorithm to compare with other sequences and to verify that sequences were of the target group. Sequences were aligned manually using MacClade (Maddison and Maddison, 2002). Ambiguously aligned regions were excluded from the analyses. Phylogenetic analyses were conducted using maximum parsimony and maximum liklihood methods. Parsimony analyses were carried out using a heuristic search in PAUP 4.0b10 with 1000 random addition sequences and 5000 bootstrap replicates (Swofford, 2001). Two independent maximum likelihood analyses based on a general-time-reversible 6-parameter model of evolution were run using the software program GARLI and included 100 bootstrap replications (Zwickl, 2006).

Results

Molecular analyses of LSU and SSU data support the conclusions of O'Donnell *et al.* (1997) that there are distinct northern hemisphere lineages in the Tuberaceae, and distinct southern hemisphere lineages (Fig.3). Four sourthern hemisphere clades were resolved including (*Dingleya*, *Labyrinthomyces*, *Reddellomyces*, and *Leppia* nom. prov.) (Trappe and Claridge, 2005). Resolved and supported Northern hemisphere clades include *Choiromyces* and *Tuber*, *Choiromyces* is represented by *C. aveolatus* and *C. venosus*, while *Tuber* is much more speciose.

Multigene analyses of LSU, EF1a, 5.8S, and RPB2 loci support 7 clades (*Aestivum*, *Melanosporum*, *Rufum*, *Canaliculatum*, *Gibossum*, *Puberulum*, *Maculatum*) within *Tuber* (Fig.4). *Tuber asa* and *Tuber excavatum* were not well resolved in these analyses. The most basal clade (Aestivum) was composed of strictly European taxa (*T. mesentericum*, *T. aestivum*, *T. magnatum*). The *Melanosporum* (Fig.5) and *Rufum* (Fig.6) clades were reprentated by European, Asian, and North American taxa, and (aside from *T. pseudoexcavatum*) are composed of spiney and spiney-reticulated spored species. In addition, we found evidence from sequences derived from fruitbody and

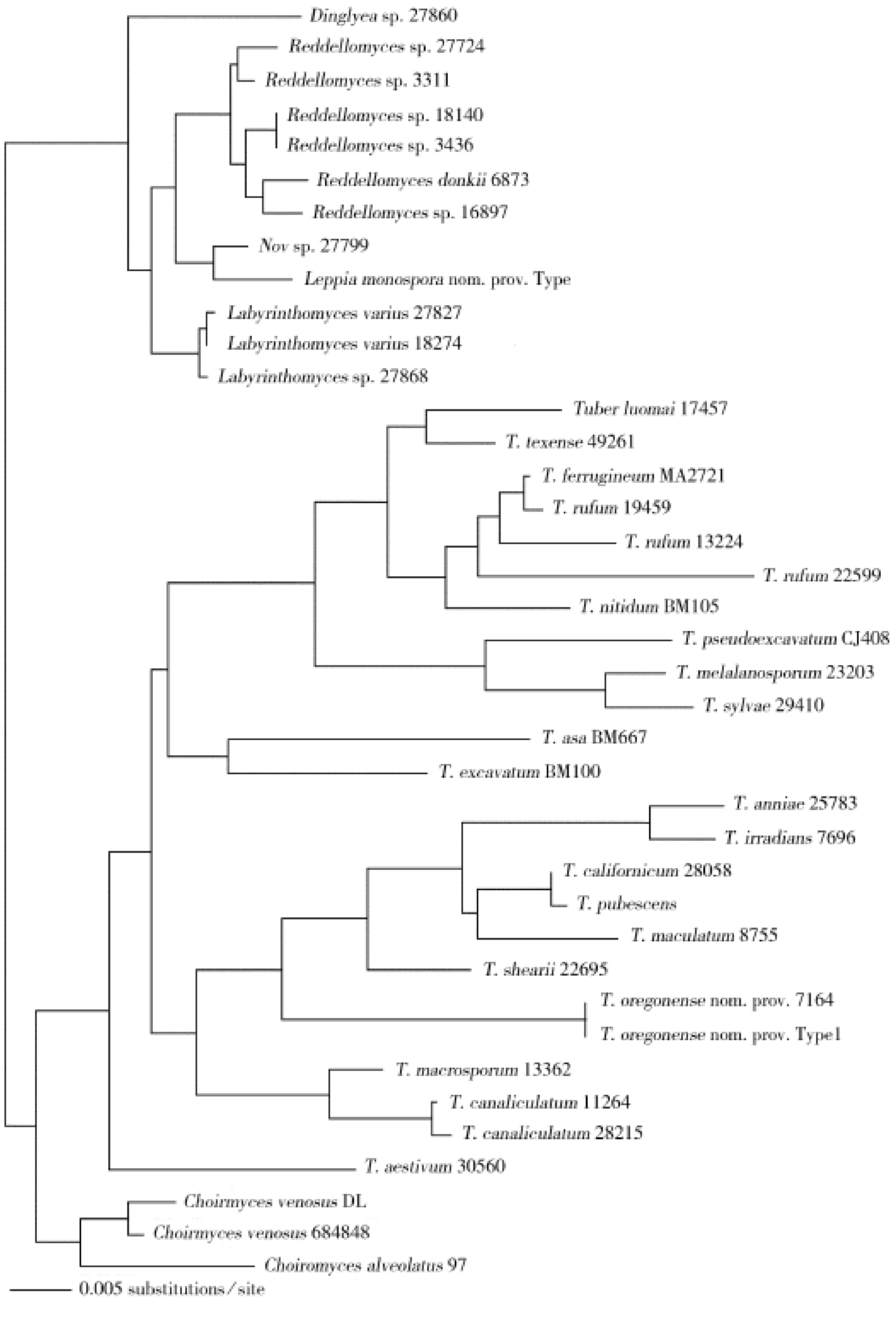

Fig . 3 Phylogeny of Tuberaceae

The Tuberaceae is represented by four Southern Hemisphere genera (*Dinglyea* , *Reddelomyces*, *Labyrinthomyces*, and *Leppia* nom . prov .) and two Northern Hemisphere genera (*Tuber*, *Choiromyces*) . This phylogenetic analysis is based on the small subunit (SSU) and large subunit (LSU) rDNA sequence data, and was analyzed using unweighted maximum parsimony

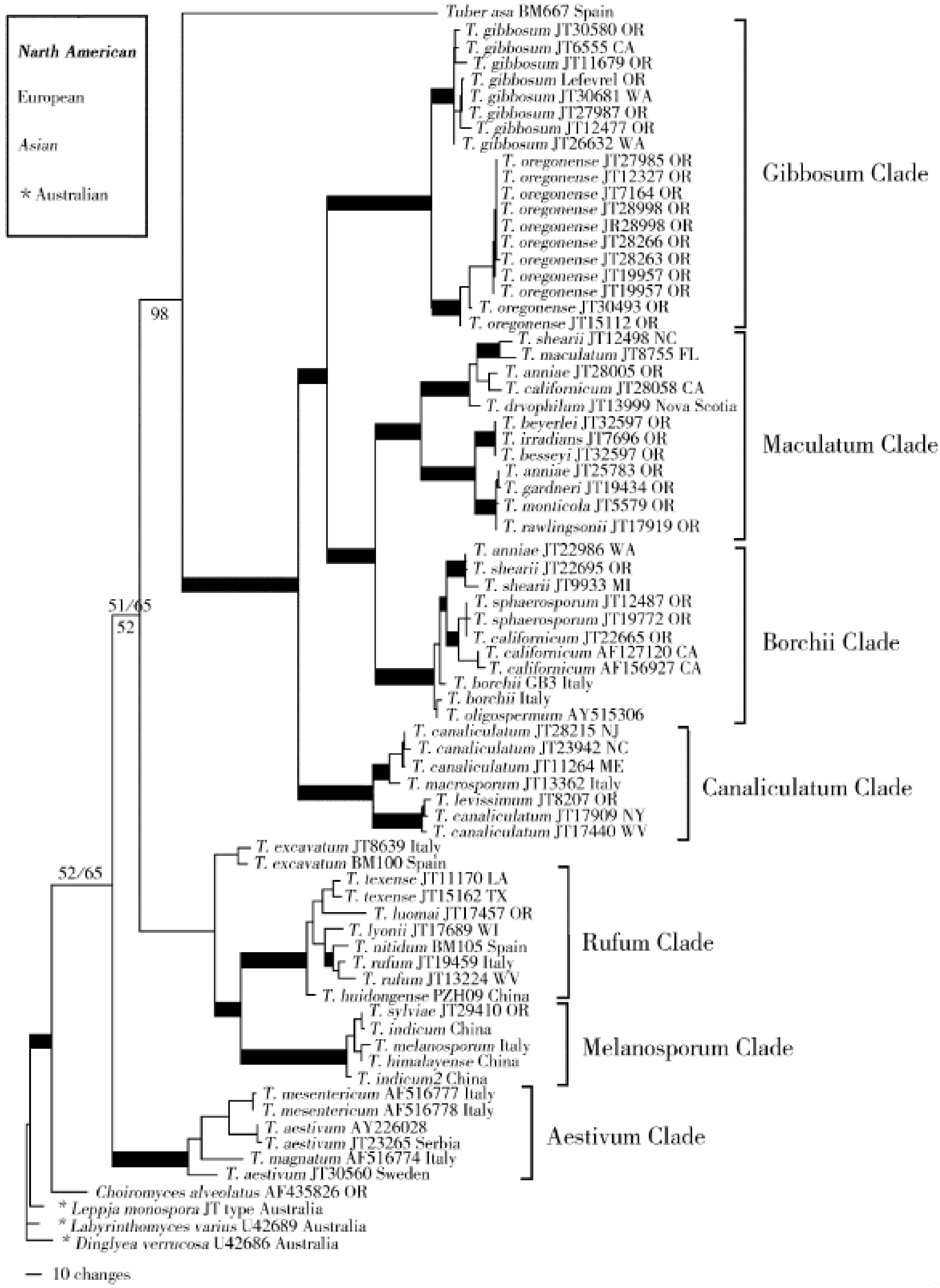

Fig . 4 Phylogeny of *Tuber*

Seven clades of Tuber were resolved with unweighted parsimony analyze based on multiple loci (rDNA LSU, 5.8S rDNA, RNA polymerase II, and elongation factor alpha 1) . These clades are the *Aestivum*, *Melanosporum*, *Rufum*, *Canaliculatum*, *Gibbosum*, *Maculatum*, *Puberulum* (*Borchii*) groups . The European species *Tuber excavatum* and *T*. *asa* were not resolved into any of these clades in this analysis . Nodes with significant support (> 70% parsimony bootstrap) are signified by thickened branches

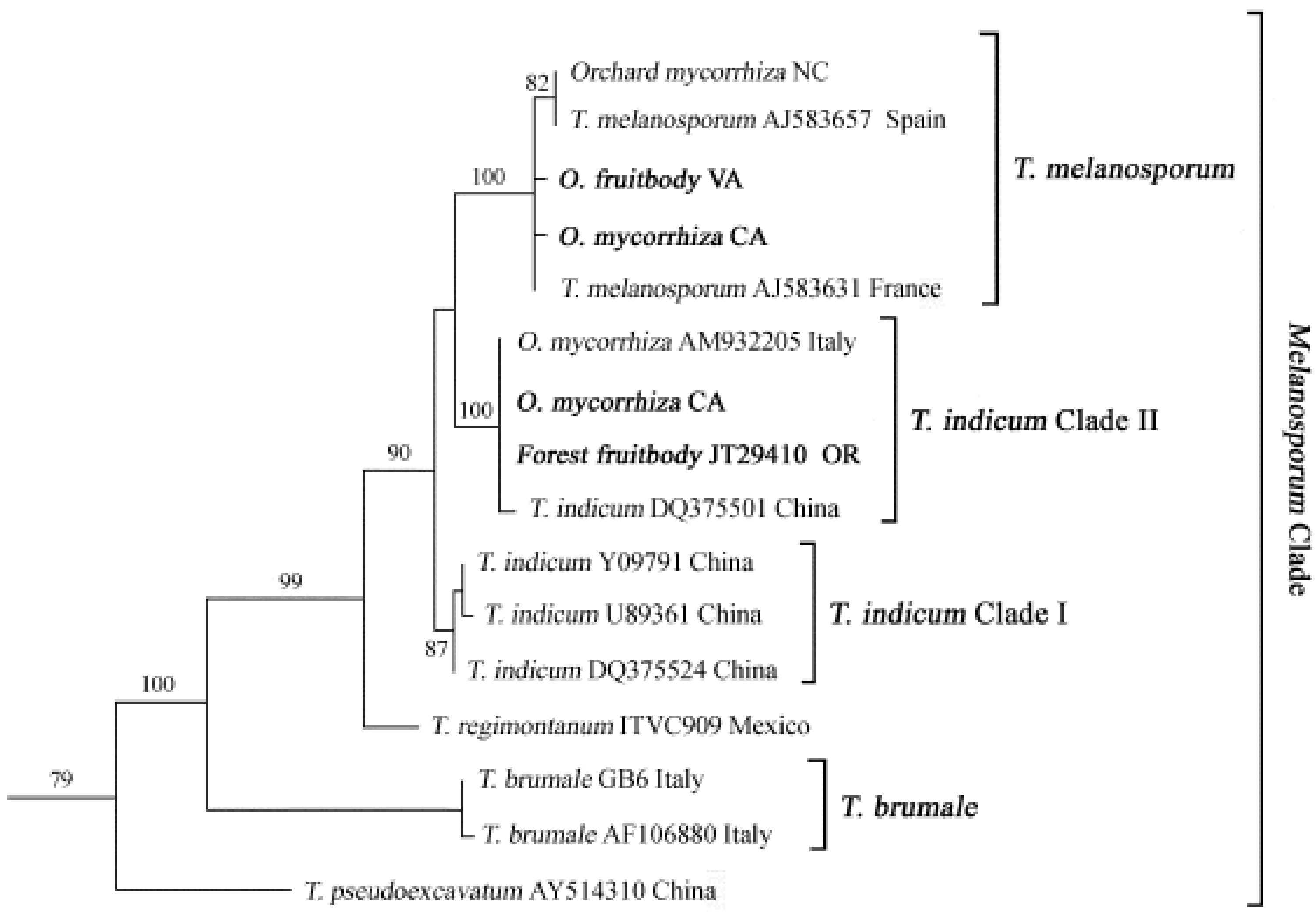

Fig. 5 The Melanosporum Clade

Representatives of the Melanosporum clade in North America include *Tuber regimontanum*, *T. melanosporum*, and *T. indicum*. *Tuber melanosporum* has been introduced in efforts to cultivate this species as a cash crop. It is most likely that *T. indicum* (clade II) was introduced into North America through forestry projects early in the century (e.g. fruitbody collection) and more recently (accidentally) through *T. melanosporum* cultivation efforts. These three species are similar in morphology, though *T. regimontanum* has larger spores than *T. melanosporum* and *T. indicum* and short reticulations connecting the spines of its spores. Results are based on parsimony analysis of the ITS rDNA region. Taxa names are followed by collection (or Genbank accession) numbers, followed by point of origin (when known)

mycorrhizas suggesting that the Asian species *T. indicum* has been introduced into the United States. Two species were resoloved in the *Canaliculatum* clade; *T. macrosporum* (Europe) and *T. canaliculatum* (North America), while the *Gibbosum* clade was composed of soley of species restricted to the Pacific Northwest region of North America (Fig.7). The *Puberulum* (Fig.8) and *Maculatum* (Fig.9) clades were represented by taxa from Asia, Europe, and North America, and together with the *Rufum* clade includes the majority of *Tuber* species. They present a taxonomic challenge to mycologists, owing to a lack of apparent morphological characters for distinguishing species from one another.

Conclusions

Europe appears to have the most phylogenetic diversity of *Tuber*, particularly represented by basal lineages, suggesting that this continent may be the point of origin for the genus. However *Tuber* diversity in Asia is still not well known and the discovery and inclusion of new taxa in future analyses could change inferences about the origin and diversification of *Tuber*.

Further work should focus on molecular analysis of type specimen (when possible) and attempt a more comprehensive coverage of the species diversity within the family Tuberaceae. In addition, it is still undetermined whether the genus *Paradoxa* and *Loculotuber* fall within the Tuberaceae, and if so, whether these genera are mono-, para-, or polyphyletic.

Fig. 6 The Rufum Clade

North America is well represented by spiney-spored members of the Rufum clade. As far as we are aware, *T. rufum* Vittadini senso stricto does not exist in Asia or North America. Results are based on parsimony analysis of the ITS rDNA region. Nodes with significant support (> 70% parsimony bootstrap) are signified by thickened branches. Taxa names are followed by collection (or Genbank accession) numbers, followed by point of origin (when known). Names in quotations are from collection labels and are most likely incorrect

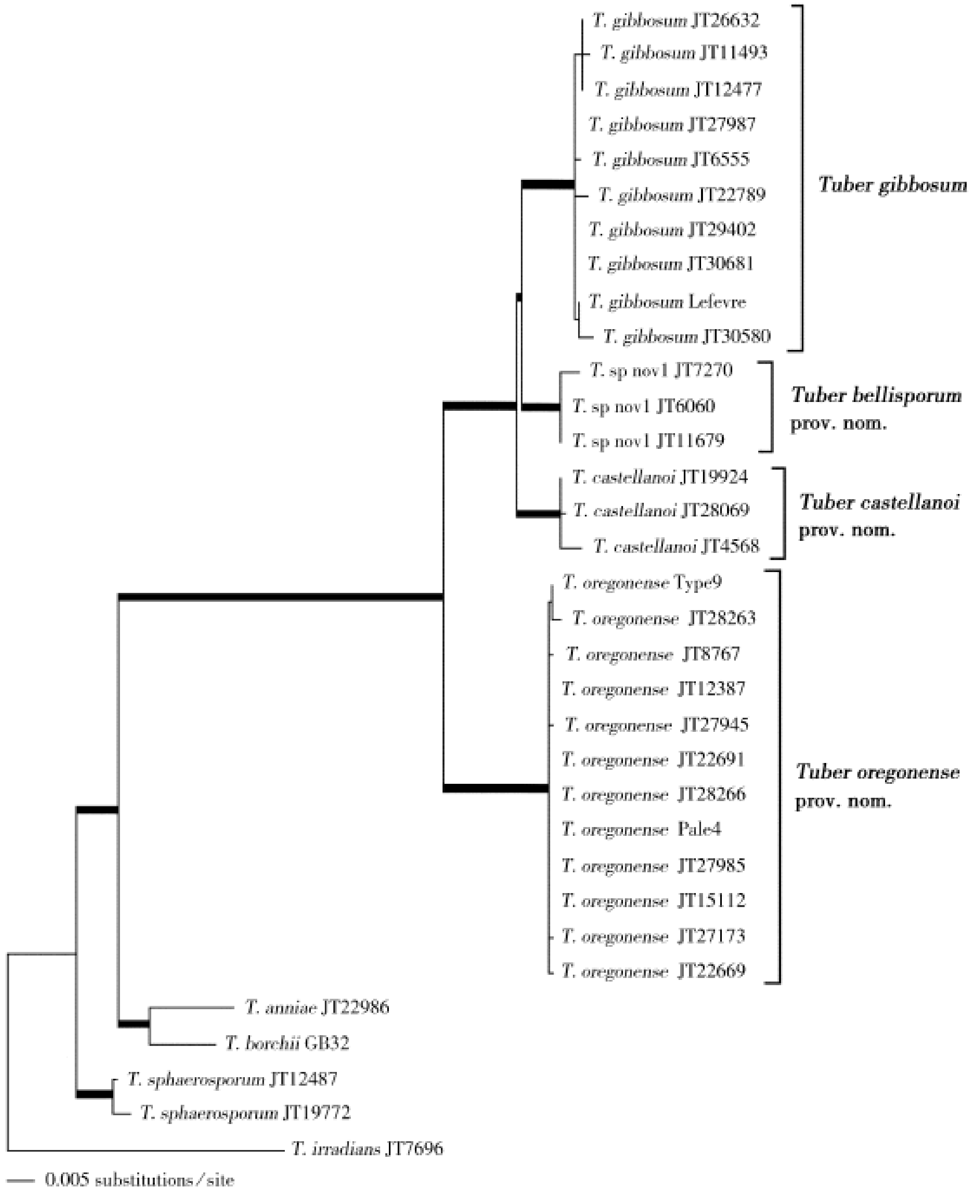

Fig . 7 The Gibbosum Clade

To the best of our knowledge members of the Gibbosum clade are only represented in the Pacific Northwest region of North America . The four species include *T*. *gibbosum*, *T*. *bellisporum* prov . nom ., *T*. *castellanoi* prov . nom ., and *T*. *oregonense* prov . nom . . Results are based on maximum liklihood analysis of the ITS and LSU rDNA regions . Nodes with significant support (> 70 % maximum liklihood bootstrap) are signified by thickened branches . Taxa names are followed by collection numbers

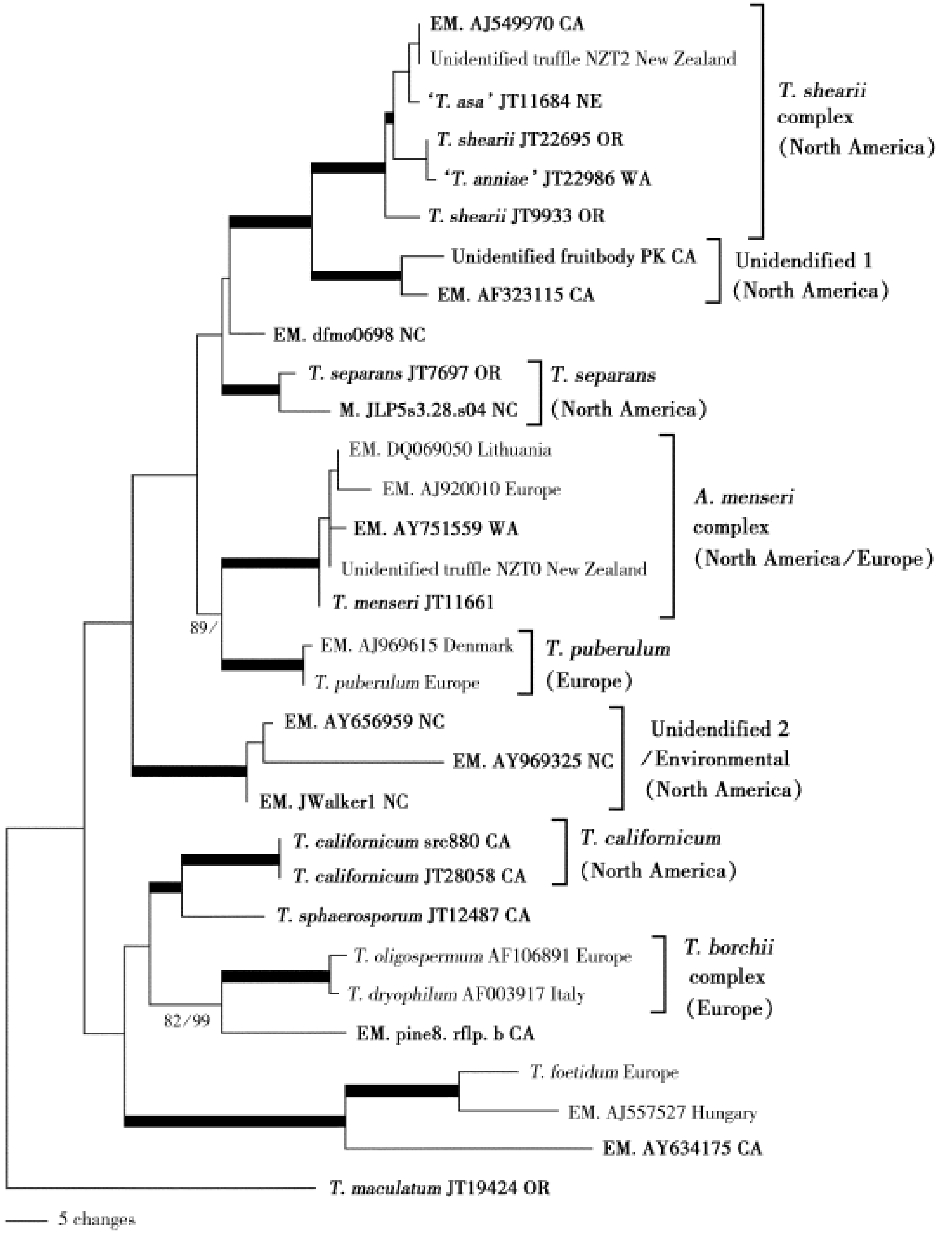

Fig . 8 The Puberulum Clade

Many undescribed and unidentified species (resulting from mycorrhizal molecular studies) belong to the Puberulum clade . Morphologically, this group tends to have globose to sub-globose spores, and fruitbodies that are pale in color and small in size . Species in this group are found in Europe, North America, and Asia . Results are based on parsimony analysis of the ITS rDNA region . Nodes with significant support (> 70% parsimony bootstrap) are signified by thickened branches . Taxa names are followed by collection (or Genbank accession) numbers, followed by point of origin (when known) . Names in quotations are from collection labels and are most likely incorrect

Fig. 9　The Maculatum Clade

Many undescribed and unidentified species (resulting from mycorrhizal molecular studies) belong to the Maculatum clade. Morphologically, this group tends to have sub-globose to elliptical spores, and fruitbodies that are pale in color and small in size. Species in this group are found in Europe, North America, and Asia. Results are based on parsimony analysis of the ITS rDNA region. Nodes with significant support (> 70% parsimony bootstrap) are signified by thickened branches. Taxa names are followed by collection (or Genbank accession) numbers, followed by point of origin (when known). Names in quotations are from collection labels and are most likely incorrect

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